Seasonal variations in the functional performance of industrial low-moisture part-skim Mozzarella over a 1.5 year period

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Key Words:	low-moisture part-skim Mozzarella, cheese characteristics, principal component analysis, process variability

INTERPRETIVE SUMMARY

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Title: Seasonal Variations in the Functional Performance of Industrial Low-Moisture Part-Skim Mozzarella over a 1.5 year Period

Low-moisture part-skim Mozzarella is used in the pizza-industry as a cheese topping because of its desirable melt and stretch properties upon cooking. The current study investigated the relationships between the physicochemical and functional properties of low-moisture part-skim Mozzarella, which was manufactured according to a standardized procedure on industrial scale. In addition, specific attention was paid to the variability in the functional performance of the cheese during storage. Our study identified variation in calcium content of the cheese as the major factor responsible for vat-to-vat variation in cheese quality. We recommend a tight control of the calcium level to obtain a more consistent quality.

13 VARIATIONS IN QUALITY OF INDUSTRIAL MOZZARELLA

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15 **Seasonal variations in the functional performance of industrial low-moisture part-**

16 **skim Mozzarella over a 1.5 year period**

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ABSTRACT

Seventy-five blocks of low-moisture part-skim (**LMPS**) Mozzarella cheese were procured from an industrial cheese plant, and the relationships between the physicochemical and functional properties were evaluated during refrigerated storage. In total, cheeses were obtained from 1 cheese vat on 7 different production dates, at two- to four monthly intervals, over a 1.5 year period; all cheeses were made using a standard recipe. The cheeses were held at 4°C for 0, 1, 2, 4, 8, 16 or 32 d and assayed for composition, primary proteolysis, serum distribution, texture profile analysis, heat-induced changes in viscoelastic behavior, cheese extensibility and melt characteristics. The results demonstrated a substantial increase in serum uptake by the calcium-phosphate para-casein matrix between 1 and 16 d of storage with a concomitant improvement in the functional performance of the cheese. Extending the storage time to 32 d resulted in further changes in the functional quality, concurrent with ongoing increases in protein hydration and primary proteolysis. Differences in the measured characteristics between the cheeses obtained on different sampling occasions were evident. Principal component analysis separated the cheeses based on their variance in functional performance, which was found to be correlated mainly with the calcium content of the cheese. The results indicate that the manufacturing process should be tightly controlled to minimize variation in calcium content, and enhance the quality consistency of the cheese.

Key Words: low-moisture part-skim Mozzarella, cheese characteristics, principal component analysis, process variability

INTRODUCTION

Low-moisture part-skim Mozzarella (**LMPS**) is a plasticized cheese, with a structure typically described as a fibrous calcium-phosphate para-casein network with occluded pools of serum and fat (Oberg et al., 1993). Plasticization is typically achieved by displacing the contiguous planes of the fat-filled calcium-phosphate para-casein matrix of the fermented curd (pH ~5.1 to 5.3) by heating (~58 to 62°C), kneading and stretching the curd in hot water or dilute brine (Fox et al., 2017). The supplied thermomechanical energy alters the interactions between proteins and bestows the cheese with the ability to form extendable strands and undergo limited oiling-off when subsequently baked on a pizza (McMahon and Oberg, 2017; Sharma et al., 2016).

According to several researchers, understanding the development of the curd structure during manufacturing and the overall interactions between the para-casein fibers could help in optimizing the functional quality of the cheese (Oberg et al., 1993; Feeney et al., 2001; Lucey et al., 2003). These linkages between facing casein polymers include calcium phosphate bridges between colloidal calcium phosphate and phosphoserine residues, calcium bridges between calcium ions and dissociated amino acid residues, and hydrophobic attractions between hydrophobic protein domains (McMahon et al., 1999; Lucey et al., 2003; Fox et al., 2017). The overall balance of interactions is likely to be affected by many factors including cheese composition (e.g., pH, content of protein, moisture, fat and calcium), degree of proteolysis, shear work input during curd stretching and curd temperature during manufacturing, storage and cheese application. Smith et al. (2017) investigated the effects of refrigerated storage on the microstructure of a 10 kg industrial Mozzarella cheese block, and demonstrated how changes in hydrophobic interactions and proteolytic breakdown were significant drivers in the alteration of the calcium-phosphate para-casein matrix. In particular, free moisture, as measured by ^{1}H-NMR relaxometry, was gradually taken up by the para-casein matrix within 20 days after manufacturing of the cheese, with a

concomitant weakening of the hydrophobic interactions. These results were in agreement with those of Kuo and Gunasekaran (2009) and McMahon et al. (1999) who investigated the microstructure of LMPS Mozzarella cheeses during 14 or 21 d storage at 4°C using scanning electron microscopy, and described how the uptake of moisture resulted in swelling of the calcium-phosphate para-casein matrix, and the formation of a reticular network of distinctly defined flat globules occluded by the para-casein matrix. Similarly, Gianferri et al. (2007) reported substantial displacement of 'serum water', described as the accumulated water in protein fiber channels, to 'junction zone water', described as the water that could be seen as an integral part of the protein structure, in retail Mozzarella di Bufala Campana cheese (moisture content of 55 to 60%, wt/wt) during storage at 8°C for 7 d.

Other studies focused on the biochemical and thermophysical (functional, baking) properties of LMPS Mozzarella during refrigerated storage. Increasing storage temperature of LMPS Mozzarella from 0 to 15°C over a 70 d period resulted in more rapid depletion of expressible serum during the first 20 d, higher primary proteolysis and flow over 70 d, and higher extensibility after 5 to 20 d (Feeney et al., 2001; Guinee et al., 2001). Similar effects of storage time on the functional performance have been reported for LMPS Mozzarella cheeses, manufactured on pilot-scale (Guinee et al., 2002; Dave et al., 2003; Imm et al., 2003; Banville et al., 2013).

Previous studies (Smith et al., 2017; Vermeir et al., 2019) evaluated storage-related changes in industrial LMPS Mozzarella using ^{1}H-NMR relaxometry, but did not relate these changes to the concurrent changes in other physicochemical or functional characteristics. The objectives of the current study were firstly to establish the relationships between the biochemical, water-distribution, and functional characteristics in industrial LMPS Mozzarella cheeses during storage at 4°, and secondly to monitor inter-vat variability in these characteristics between the cheeses sampled on 7 different production dates over a 1.5 year period. Identifying the source of this variability could

afford manufacturers with a clearer view of the factors that impact quality, and thereby assist the production of cheese with a more consistent quality.

MATERIALS AND METHODS

Manufacturing of LMPS Mozzarella

LMPS Mozzarella was manufactured at Milcobel CV (Langemark, Belgium) following a standardized procedure. Milk was standardized to a fat content of 2.8% (wt/wt), pasteurized, cooled to 35°C, pumped to the cheese vat, and inoculated with a freeze-dried culture (*Streptococcus thermophilus*). $CaCl_2$ (33%, wt/wt) was added to a final concentration of 0.42 mM Ca. A commercial liquid microbial coagulant EC 3.4.23.23 (endopeptidase derived from *Rhizumucor miehei*) was dosed at a level of 23.83 $IMCU.L^{-1}$ milk. The milk was allowed to stand for approximately 30 min during which time it gelled. The gel was cut into curd grains, and the curd-whey mixture was stirred for ~20 min, and cooked at 39°C for ~20 min. After curd cooking, the whey was drained and the curd grains were collected on a transport belt and held at 35°C to promote curd dehydration and acidification, which resulted in fusing of the curd grains into a consolidated curd mass. When the curd attained a pH of 5.05 to 5.25, it was milled, diced, heated to 62°C using a water-steam mixture, and kneaded and stretched mechanically into hot uniform molten mass. The stretched curd was extruded into a mat, sprinkled with salt (0.9%, wt/wt), moulded into blocks (2.5kg; 28 cm x 10 cm x 8 cm) and held in a brining bath at 4°C until the core of the cheeses was cooled down to 4°C. After brining, the cheese blocks were rinsed with water, packaged and stored at 4°C.

Experimental Analysis

Production. Every two to four months over a period of 16 months (November 2017 - February 2019), a cheese vat (coded A, B, C, D, E, F and G, resp.) was sampled to determine the effects of

variability in milk composition, cheese pH at different stages of manufacture, and cheese composition on the functional characteristics of the cheese. Every production was done with the same thermophilic starter culture and commercial rennet, and the process was operated under standardized conditions (time, temperature and amount of water-steam mixture injected during curd plasticization).

Sampling. For each production date, the milk was stirred and sampled before rennet was added. A sample of the curd-whey mixture was collected during whey drainage, such that the sample corresponded to the middle of the cheese vat. A portion of the fermented curd was sampled from the transport belt before milling, such that the sample corresponded to the middle of the cheese vat. The milk, curd-whey and curd samples were analyzed for pH (Knick, 765 Laboratory pH meter, SE 503 pH sensor, Berlin, Germany) directly after sampling; the results were denoted as $\mathbf{pH_{renneting}}$, $\mathbf{pH_{drainage}}$ and $\mathbf{pH_{milling}}$, respectively. The milk samples were stored at 4°C for less than 8 h and analyzed for fat and protein (Milcoscan FT2, Foss, Hilleroed, Denmark).

Consecutive cheese blocks were removed at the end of the production line, such that the sampled blocks corresponded to the middle of the cheese vat. This was chosen to minimize the inter-block variability between cheeses taken from the same vat. After sampling, the cheeses were sealed in plastic vacuum bags, and stored at 4°C for up to 32 d. A total of 75 industrial cheese blocks were sampled. An overview of the sampling pattern of the blocks per production date and per storage time is given in Table 1. The serum distribution was evaluated in separate cheese blocks due to the difference in location between the analytical instruments.

Cheese Composition. Grated LMPS Mozzarella was analyzed for moisture, fat, total N, salt, Ca and pH, as described previously (To, et al., 2020). Moisture, fat and pH were measured in duplicate on each cheese block. Total N, salt and Ca were measured in duplicate on each cheese block from vat A during storage. For the subsequent cheese vats (vats B, C, D, E, F and G), the contents of

total N, salt and Ca were analyzed in duplicate on at least four cheese blocks after 2 or 4 d of storage.

Soluble Ca and pH 4.6 Soluble N. The levels of soluble Ca and pH 4.6 Soluble N (**pH4.6SN**) were determined on a water-soluble extract of the cheese, as described previously by To et al. (2020). Serum-soluble Ca was expressed as a percentage of the total cheese Ca content and pH4.6SN was expressed as a percentage of total cheese N. Measurements were performed in duplicate per cheese block.

Time Domain ^{1}H NMR Relaxometry. The T_2 relaxation time distribution of LMPS Mozzarella was evaluated by low-field NMR on a benchtop Maran Ultra spectrometer (Oxford instruments, Abingdon, UK), operating at 0.55T (23.4 MHz for ^{1}H). The method was described by Vermeir et al. (2019) who distinguished three serum fractions comprising liquid oil protons and water protons in LMPS Mozzarella with different T_2 relaxation times. The serum fraction characterized by a $T_2 >$ 60 ms was ascribed to weakly interacting serum protons and could be interpreted as 'more-mobile-serum', whereas the other serum fractions, characterized by $T_2 < 3$ ms or $T_2 \approx 10$ ms, were interpreted as 'less-mobile-serum'. In this study, the integrated signal intensity of the less-mobile-serum fractions, $\mathbf{A_{3ms}}$ and $\mathbf{A_{10ms}}$, and the more-mobile-serum fraction, $\mathbf{A_{60ms}}$, were reported. A_{3ms} and A_{10ms} were indicative of serum that interacted with the calcium-phosphate para-casein network of the cheese, whereby shorter T_2 times indicated stronger interactions. Triplicate measurements were performed at two separate locations in one Mozzarella block after 0, 1, 2, 4, 8, 16 or 32 d storage at 4°C, sampled from vats A, B or G (Table 1). The data were used to monitor the behavior of cheese serum during refrigerated storage and its relation to the functional performance of the cheese.

Functional Properties. The methods for assaying the functional performance of the cheese have been described previously in detail (To et al., 2020), and are summarized below.

Texture Profile Analysis. Cheese cubes (25 mm x 25 mm x 25 mm) were loaded individually on a TAHdi texture analyzer fitted with a 100 kg load cell (Stable Micro Systems, Goldalming, UK), and compressed in two consecutive bites at a speed of 1 $mm.s^{-1}$ to 60% of its original height. The following characteristics were reported: maximum compression force recorded during bite 1 (firmness), the ratio of height to which the cube was compressed at the start of bite 2 relative to the sample's original height (springiness), the ratio of work required to compress the cube in bite 2 relative to that of bite 1 (cohesiveness) and the product of firmness x springiness x cohesiveness (chewiness). Measurements were performed in sextuplicate per cheese block.

Extensibility of the Heated Cheese. Shredded cheese was heated to 95°C and the molten curd (85-95°C) was loaded on a TAHDi texture analyzer (Stable Micro Systems, Goldalming, UK) and uniaxially extended at a rate of 10 $mm.s^{-1}$ to a height of 380 mm. Extension work (**EW**) was defined as the cumulative work required to extend the hot molten cheese, directly after heating (**EW_0**) and after allowing the cheese to cool down for 5 minutes at room temperature (**EW_5**). EW_0 and EW_5 were measured in triplicate and in duplicate per cheese block, respectively. EW_5 was used to simulate the impact of cooling-induced stiffening of molten cheese on a pizza during consumption and was assessed for cheeses sampled from vats C, D, E, F and G.

Heat-induced Changes in Viscoelastic Behavior. Cheese discs (50 mm diameter; 2 mm thickness) were placed between parallel cross-hatched plates (PP50/P2-SN27902; INSET I-PP50/SS/P2) on a strain-controlled rheometer (MCR501, Anton Paar GmbH, Graz, Austria), subjected to a low amplitude shear strain ($\gamma = 0.0063$) at an angular frequency of 1 Hz and heated from 25°C to 90°C. The cross-over temperature (**COT**), corresponding to the point at which the

cheese transitioned from the solid phase into the liquid phase, and the maximum value of the loss tangent (**LT_{max}**), an index for the fluidity of the cheese during heating, were reported. Measurements were performed in duplicate per cheese block.

Flow. Cheese discs (45 mm diameter; 4 mm thickness) were heated at 280°C for 4 minutes in a convection oven (Binder FD 35, Binder GmbH, Tuttlingen, Germany). Flow was defined as the percentage increase in mean diameter during heating. Measurements were performed in quadruplicate per cheese block.

Statistical Analysis

LMPS Mozzarella was manufactured on seven different production dates according to a standardized procedure. The cheeses from the different production dates were analyzed for changes in physicochemical and functional characteristics during storage, and the data were analyzed using a randomized incomplete block design incorporating storage time as the treatment and 7 blocks (replicate trials). JMP 14 statistical software package was used to determine significant differences between mean compositional values of cheeses obtained from different vats by One-way ANOVA and Tukey's HSD post-hoc test.

The relationships between the physicochemical (A_{3ms}, pH, serum-soluble Ca and pH4.6SN) and functional (firmness, COT, LT_{max}, EW_0 and flow) characteristics of the cheeses during storage at 4°C were evaluated using simple linear regression analysis, and the significance was determined by application of an F-test with n – 2 df.

The variability in the characteristics of the cheeses during storage at 4°C was evaluated using principal component analysis (**PCA**), which identifies linear combinations of correlated variables, i.e., principal components, while retaining the highest amount of variability among the studied

variables. First, the Kaiser-Meyer-Olkin (**KMO**) test and Bartlett's test of sphericity were used to test the sampling adequacy and the degree of correlation between variables, respectively. The KMO test measures the proportion of variance that could be attributed to underlying principle components whereas the Bartlett's test of sphericity tests the correlation matrix against the identity matrix. PCA was performed when the KMO value was larger than 0.6 and when the Bartlett's test of sphericity returned significant at $P < 0.05$, and the minimal number of principal components was derived based on Eigenvalues larger than 1, Skree plot analysis and the cumulative percentage of variance explained. Varimax rotation was used to obtain principal components that were not correlated, and to reduce the amount of variables.

Two-way ANOVA was then used to determine the effects of variations in the composition, storage time at 4°C and their interactions on the functional performance-related components, separately. The level of significance was determined at $\alpha = 0.05$ throughout. For each functional performance-related component, the 'fit model' function of JMP 14 was used to fit a linear model to the variables that were found to have a significant effect.

RESULTS AND DISCUSSION

In-line Process Analysis

The mean fat and protein content of the milk used for cheese-making was 2.83% and 3.67%, respectively. The variability in the contents of fat and protein at the seven different production dates over the 1.5 year period is indicated by the difference between the maximum and minimum value of the measured data, divided by the average (Table 2), and reflects the width of the distribution for a given average on the condition that the data is normally distributed and no significant outliers are present. The milk protein content showed a variability of 8.4% in the current study, which was consistent with the data reported by Eurostat (2020), which reported a variability of 7.6% in the protein content (in non-fat dry matter) of Belgian raw milk for the year 2019, and reflects the natural variation associated with differences in breed, feed type, season or stage of lactation. Walstra et al. (2006) reported that the fat content in raw milk showed the highest variability among all milk constituents. As legal "Standards of Identity" impose FDM specifications on many cheese varieties, this highlights the importance of fat standardization for cheese-making (Fox et al., 2017).

The curd becomes suitable for plasticization when sufficient Ca is released from the calcium-phosphate para-casein network, and is governed by the values for $pH_{renneting}$, $pH_{drainage}$ and $pH_{milling}$ which upon decrease promote solubilization of colloidal Ca by displacement with acidic protons (Fox et al., 2017; McMahon and Oberg, 2017). The former two values are critical for mediating the total calcium content of the curd, and hence the pH of the curd at which sufficient Ca is released (Kindstedt et al., 2004; McMahon and Oberg, 2017). Typical $pH_{drainage}$ and $pH_{milling}$ values of 6.20 to 6.00 and 5.15 to 5.30 are reported, in respective order, for the production of LMPS Mozzarella (Fox et al., 2017; McMahon and Oberg, 2017). The mean values for $pH_{renneting}$, $pH_{drainage}$ and $pH_{milling}$ over the seven cheese-making days in the present study were 6.54, 6.37 and 5.15 for this

particular long shelf life recipe, respectively. The average $pH_{drainage}$ applied by the plant in this study was relatively high, and a $pH_{milling}$ near the lower limit was required in order to plasticize the curd. The mean Ca content of the resulting cheeses in this study (29.9 mg $Ca.g^{-1}$ protein) was relatively high when compared to those reported by Guinee et al. (2000), who evaluated the composition of 8 commercial low-moisture Mozzarella cheeses (22.6 to 31.1 mg $Ca.g^{-1}$ protein), and thus reflects the wide range of $pH_{renneting}$, $pH_{drainage}$ and $pH_{milling}$ applied in commercial practices. All cheeses conformed to the specifications of dry matter (**DM**) and fat-in-dry matter (**FDM**) for low-moisture part-skim Mozzarella, as defined by the Code of federal regulation (48%, w/w < DM < 55%, w/w; 30%, w/w < FDM < 45%, w/w) (FDA, 2020). The mean values for the contents of DM, FDM, moisture-non-fat-substances (**MNFS**), salt-in-moisture (**S/M**) and Ca differed between production dates at $P < 0.05$, with S/M and Ca showing the highest variability. Variability likely reflects differences in milk composition (levels of protein, fat, lactose and Ca; pH) and manufacturing process (ratio of added rennet and starter culture to milk casein content, starter culture activity, pH at different stages of manufacture) across the cheesemaking season (Chen et al., 2014; Lin et al., 2017; Gulati et al., 2018, 2019). Such factors predetermine the composition and, ultimately, the biochemical and functional properties of the cheese (Fox et al., 2017). The relatively high variability between different production dates for Ca and S/M is consistent with that previously reported for industrial Mozzarella and Cheddar cheeses (Guinee et al., 2000), which may be associated with differences in rate of acid development and curd pH profile at different stages of manufacture. Acid development, which is influenced by the interactive effects of many compositional and cheesemaking variables, is a key determinant for calcium release from, and salt uptake by, curd on transit through the manufacturing process (Fox et al., 2017).

Overall Changes during Storage at 4°C

The storage-related changes in physicochemical and functional characteristics of cheese across the seven different production dates are shown in Figure 1. The data for the individual vats at different storage times are available in Supplementary Table A.

Physicochemical Changes. Increasing storage time resulted in a reduction in more-mobile-serum (A_{60ms}) ($P < 0.001$) and increases in less-mobile-serum (A_{3ms} and A_{10ms}) ($P < 0.001$), pH4.6SN ($P < 0.001$) and pH ($P < 0.05$). The trends concur with those of previous studies (Guo and Kindstedt, 1995; Guinee et al., 2002; Smith et al, 2017). Smith et al. (2017) suggested that the reduction in A_{60ms} was related to the increase in soluble Ca content during early storage (e.g., 20 d; Guo and Kindstedt, 1995; O'Mahony et al., 2006), concomitant with the solubilization of colloidal calcium phosphate (**CCP**), and hypothesized that the CCP solubilization would expose the highly polar phosphoserine groups of the casein and thereby promote water immobilization through hydrogen bonding. Additionally, salt in the cheese contributes to salting-in and hydration of the casein during early storage (Guo et al.,1997) due to exchange of casein-bound Ca with Na. The increase in pH is of comparable magnitude to that reported previously for LMPS Mozzarella (Guo et al., 1997; Guinee et al., 1998), and has been attributed to a storage-related increase in the solubilization of CCP and the pH-upward buffering effect associated with the protonation of the released phosphate groups (HPO_4^{2-}) in the serum phase (Upreti et al., 2006; Smith et al., 2017).

Storage time did not affect the proportion of serum-soluble Ca at $P > 0.05$, which varied from 28% to 43% of total Ca (Fig. 1). This trend contrasts with that of Guo and co-workers (Guo and Kindstedt, 1995; Guo et al., 1997) who found a significant increase in the concentration of Ca in serum expressed from LMPS Mozzarella cheese on centrifugation at 12,500 *g* over the first 8 d of storage; simultaneously, the volume of expressible serum decreased. However, the current results agree with those of Metzger et al. (2001) who showed that the soluble Ca content (as % of total Ca in cheese) of a water-soluble extract of LMPS Mozzarella, prepared by filtration of a cheese-water

homogenate, changed little from 40 to 45% over the course of a 90 d storage period at 4°C. The discrepancy between these findings may be attributed to variations in curd temperature at stretching. Metzger et al. (2001) applied a similar temperature at curd stretching (62.3°C) to that in the current study (~62°C), whereas Guo and Kindstedt (1995) and Guo et al. (1997) stretched the curd at a lower temperature (54°C). These stretching temperatures were in accordance with the values reported for commercial practices (50 to 65°C) (Renda et al., 1997), and may differ depending on the intended markets. For example, higher stretch temperatures may be applied to render the cheese more firm during storage through greater inactivation of the starter culture and residual coagulant activity (Feeney et al., 2001). Kindstedt et al. (1995) increased the plasticization temperature from 62 to 66°C by varying the screw speed and found that higher plasticization temperatures resulted in cheeses with lower amounts of serum-soluble Ca and increased firmness during storage at 4°C, thereby indicating that a slight increase in curd temperature during stretching may already induce a shift in Ca distribution to the casein-bound state, and thus promote the aggregation of the calcium-phosphate para-casein network (Kindstedt et al., 2004). The discrepancy between results may also be attributed to differences in curd stretching time. Sharma et al. (2016) investigated the effects of shear work input during curd stretching on the viscoelastic properties of three model Mozzarella cheeses at fixed screw speeds. Longer stretching times at fixed screws speed resulted in cheeses with increased stiffness and reduced flow upon heating. The authors proposed that the cheese structure transitioned from an entangled polymer network to a highly aggregated network of casein particles with enhanced calcium bridging. It is likely that the extraction method, conditions of extraction, and in particular pH of the cheese after manufacture and changes in pH during storage further affected the proportion of serum-soluble Ca in LMPS Mozzarella (Guinee et al., 2000; Metzger et al., 2001; Hassan et al., 2004).

Functional Properties. The springiness or cohesiveness of the unheated cheese decreased ($P < 0.001$) during storage at 4°C (graphs not shown) whereas firmness remained unchanged at $P > 0.05$ (Fig. 1). These results were in contrast with those of Yun et al. (1993b), who found no effects of storage for up to 50 d at 4°C on the cohesiveness of unheated LMPS Mozzarella whereas hardness and springiness decreased significantly. The lack of storage-related effects on hardness may reflect the inter-vat variation in composition or the relatively low levels of pH4.6SN (Yun et al, 1993a). The mean values for COT, EW_0 and EW_5 decreased during storage at 4°C ($P < 0.001$) whereas those for LT_{max} and flow increased ($P < 0.001$). These trends were consistent with the increases in A_{3ms} and pH4.6SN. We found the largest changes in serum distribution (A_{3ms} and A_{60ms}) and functional performance (COT, LT_{max}, EW_0 and flow) during the first 16 d of storage after which the rate of change decreased markedly. In contrast, the pH4.6SN increased linearly over the 32 d storage. Similar trends were reported by Guinee et al. (2001) and Imm et al. (2003). Increases in protein hydration and proteolysis have been considered as factors that facilitate the displacement of the calcium-phosphate para-casein network when the cheese is subjected to heating and extension and shear stresses during baking and consumption, with consequent increases in LT_{max} and flow, and decreases in COT, EW_0 and EW_5 during storage at 4°C (McMahon et al., 1999; Guinee et al., 2001; McMahon and Oberg, 2017).

Relationships between Physicochemical and Functional Properties

The relationships between physicochemical and functional characteristics of the cheeses are illustrated in Figure 2. Despite the scatter associated with the different production dates and storage time, linear regression analysis indicated significant relationships between characteristics of the heated cheese (COT, LT_{max}, EW_0 and flow) and protein hydration (A_{3ms}), or pH4.6SN (Table 3). Additionally, protein hydration and pH4.6SN affected the springiness and cohesiveness of the

unheated cheese but did not affect the firmness at $P < 0.05$. Cheese pH varied from 5.3 to 5.6 and affected COT and LT_{max}, whereas the proportion of soluble Ca (28 to 42%) affected cheese firmness only. The analysis confirms the importance of para-casein hydration (A_{3ms}) and hydrolysis (pH4.6SN) as modulating factors on the functionality of heated LMPS Mozzarella. Typically, the increases in A_{3ms} and pH4.6SN in LPMS Mozzarella during the first 16 d of refrigerated storage are critical in transforming its melting behavior and bestowing it with acceptable functionality when baked (e.g., smooth, fluid, extensible and flowable cheese). The continued storage to 32 d promotes further changes in these factors, especially protein hydrolysis, and thereby further alters the functionality.

Overall Variability in Industrial LMPS Mozzarella

The mean values and standard deviation for the different compositional constituents are shown in Table 2, and those of the physicochemical and functional characteristics after different storage times in Figure 1. Evaluation of this variability and identifying the causative factors potentially provide cheesemakers with clearer insight into the production of cheese of more consistent functional quality.

Principal Component Analysis. PCA was used to resolve relationships between physicochemical and functional characteristics of the cheeses, and to establish a quantifiable factor to describe the overall functional performance of the cheese, which was then used to separate the cheeses on the basis of storage time and production date. First, we simplified the current multivariable study by reducing the number of functional-performance-related and cheese-make-related variables, separately (Supplementary Table B). Cheese-serum-related variables were not included in the PCA, as these were measured for 3 different production dates only (Supplementary

Table A) and sampling times did not correspond to those at which the remaining physicochemical and functional properties were evaluated.

Variation in Physicochemical and Functional Characteristics. PCA combined three physicochemical (pH, serum-soluble Ca, pH4.6SN) and eight functional (firmness, springiness, cohesiveness, chewiness, COT, LT_{max}, EW_0, flow) variables into two principal components, PC_1 and PC_2, which accounted for 58.7% and 15.8% of the total variance in the measured data, respectively. The loadings plot (Fig. 3A) illustrates how the variance in the original eleven response variables is explained by PC_1 (x-axis) and PC_2 (y-axis). The main variables contributing to a positive scoring on PC_1 are cohesiveness, springiness, EW_0 and COT whereas those contributing to a negative scoring are pH4.6SN, LT_{max} and flow. This indicated that pH4.6SN correlated positively with flow and LT_{max}, but negatively with EW_0, COT, cohesiveness and springiness. The relation between these variables was also evident from Figure 2, and is in agreement with other authors (Guinee et al., 2001, 2002; Sharma et al., 2016). The main variables contributing to a positive scoring on PC_2 were pH, firmness and chewiness, whereas soluble Ca content contributed to a negative scoring. PC_1 and PC_2 thus explain the variance in the physicochemical and functional properties of the cheese, and could be used to separate the cheeses on the basis of storage time and production date. We illustrate this in the score plot (Fig. 3B), where we group the Mozzarella blocks assayed in this study according to their scorings on PC_1 and PC_2, such that the plot was divided into four quadrants: I, II, III and IV. Cheeses in quadrant I scored positively for both PC_1 and PC_2, and thus had the highest values for pH, firmness, springiness, cohesiveness, chewiness, EW_0 and COT; such conditions would contribute to poor cheese functionality. In contrast, cheeses in quadrant III scored negatively on PC1 and PC2, and had the highest values for soluble Ca, pH4.6SN, flow and LT_{max}, which would favor an overall more desirable functionality. In conjunction with the loadings plot (Fig. 3A), Figure 3B demonstrates the effects of storage time at 4°C on the

functional performance of the cheese. Cheeses stored for 2 to 4 d at 4°C were situated mainly in quadrants I and IV, which corresponded to poor cheese functionality (positive scoring of PC_1). With further storage time (8 to 32 d), cheeses shifted gradually towards quadrants II and III and acquired a more desirable functionality as indicated by the negative scoring of PC_1, which corresponded to higher values of pH4.6SN, LT_{max} and flow, and lower values for COT and EW_0. Figure 3B thus illustrates how the functionality of the cheese improved with more negative scorings on PC_1 or PC_2, and we therefore considered the scorings on PC_1 and PC_2 as a measure for the overall functional performance of the cheese. Storage time mainly affected the cheese characteristics contributing to PC_1 (pH4.6SN, cohesiveness, springiness, EW_0, LT_{max}, COT and flow) (Fig. 1) as compared to those contributing to PC_2 (pH, soluble Ca, firmness and chewiness). For this reason, we denoted PC_1 as the 'major storage time-related' component and PC_2 as the 'minor storage time-related' component.

Figure 3B provides further insights in the variability between the cheeses sampled at the seven production dates; quadrants I and II mainly contained cheeses from production dates A, B and F, which overall had the highest scorings on PC_1 and PC_2 at each storage time. In contrast, quadrants III and IV mostly contained cheeses from production dates C, D, E or G, which had the lowest scorings on PC_1 and PC_2, and had better functional performance. We could thus trace the observed variability in functional performance of industrial LMPS Mozzarella during storage, as shown by the standard deviation in Figure 1, to these production dates (A, B and F vs. C, D, E and G) in particular, whereby the degree of variability is reflected by the distance between the different production dates in Figure 3B.

Variation in Cheesemaking Variables. PCA of the cheese-making variables, characterized by a variability greater than 1% (contents of milk fat and protein, and $pH_{renneting}$ and $pH_{milling}$) resulted in two principal components (PC_1, PC_2), which explained 94% of the cumulative variance (Fig.

3C). The variables milk protein, $pH_{renneting}$ and $pH_{milling}$ contributed mainly to a positive scoring on PC_1, and milk fat to a positive scoring on PC_2. The former indicated a positive correlation between the variables milk protein content and the $pH_{renneting}$ and $pH_{milling}$ under standardized cheese-make procedures; this reflects the importance of milk protein concentration in affecting buffering capacity and, therefore, the pH at different stages of cheese manufacture (Fox et al., 2017). We therefore accepted the linear combination of milk protein, $pH_{renneting}$ and $pH_{milling}$ into PC_1 and denoted this as the 'milk protein-related' component, and PC_2 as the 'milk fat-related' component. Figure 3D shows the resolution of the seven production dates based on the 'milk protein-related' component (PC_1) and the 'milk fat-related' component (PC_2). Overall, cheese at production dates E, F and G was produced with milk with lower fat content, relative to production dates A, B, D and C (Table 2). Cheeses from production dates B, D and E had a lower scoring for the 'milk-protein-related' component as compared to cheeses from production dates A, C, F and G. Even though the protein content of the milk in production date C was numerically lower than that at production date B (Table 2), cheeses from production date C scored relatively higher on the 'milk protein-related' component, indicating that the negative contribution of the lower milk protein content to PC_1 was partially offset by the corresponding high value for $pH_{renneting}$.

We did not subject cheese-composition-related variables (contents of DM, FDM, MNFS, S/M and Ca-to-protein) to PCA as no strong correlations could be demonstrated, with the exception of DM and MNFS.

Variability in Functional Performance of Industrial LMPS Mozzarella. The variability in 'major storage time-related' and 'minor-storage time-related' components in 59 LMPS Mozzarella blocks from seven cheese production dates after each storage time is presented in Figure 4. Overall, the variation of the 'minor storage time-related' component was higher than that of the 'major

storage time-related' component, which was indicative of a relatively large variation in pH, serum-soluble Ca, firmness or chewiness of the unheated cheese.

PCA showed that the cheeses from production dates A, B and F could be separated from those of production dates C, D, E and G based on their physicochemical and functional characteristics (Fig. 3B). The variation in functional performance between cheeses produced at different dates may be related to the variation in 'milk fat-related' component, 'milk protein-related' component or contents of FDM, MNFS, S/M and Ca. We applied Two-way ANOVA to assess the effects of these independent variables, storage time, and their interaction on the response components, separately. Variables found to have a significant effect were further analyzed using a linear model to identify which variable(s) contributed mostly to the observed variance in physicochemical and functional characteristics between the cheeses from the different production dates.

Effects of Manufacturing Variables and Cheese Composition on Cheese Functionality. The output of the two-way ANOVA is presented in Supplementary Table C. We found no interaction-effects ($P > 0.05$) between the different variables and storage time on the 'major storage time-related' or 'minor storage time-related' components. The effects of storage time on the 'major storage time-related' ($P < 0.001$) and 'minor storage time-related' ($P > 0.05$) components were discussed above.

Two-way ANOVA revealed no effects ($P > 0.05$) of the 'milk fat-related' component, FDM or MNFS on the 'major storage-time related' or 'minor storage time-related' components. This is consistent with the small difference in fat content between the cheeses (Table 2).

The 'milk protein-related' component ($P < 0.05$), S/M ($P < 0.01$) and Ca-to-protein ($P < 0.001$) affected the 'major storage time-related' or 'minor storage time-related' components; this is consistent with the data in Table 2 which showed relatively high variability for milk protein content, and for S/M content and Ca-to-protein ratio of the cheese.

We constructed a linear model to evaluate the combined effects of the 'milk protein-related' component, S/M, Ca-to-protein content and storage time at 4°C. The 'milk protein-related' component, i.e. the factor which showed the least significant effect (Supplementary Table C), returned as non-significant in the combined model and was thus removed. The resulting model revealed that Ca-to-protein content ($P < 0.01$) and storage time ($P < 0.001$) were the only factors contributing to the variability in the 'major storage time-related' component (Supplementary Table D). We therefore concluded that the variations in Ca-to-protein content of the cheese on the 'major storage time-related' component outweighed the corresponding contributions of variations in 'milk protein-related' component or S/M. The response curves (Fig. 5) show that the score on the 'major storage time-related' component of the cheese, which represents a linear combination of correlated physicochemical and functional variables (pH4.6SN, cohesiveness, springiness, EW_0, LT_{max}, COT and flow), is a function of storage time at 4°C and calcium content. PCA (Fig. 3) showed that more negative scores coincide with more desirable functional characteristics (higher LT_{max} and flow, lower COT). Hence, the storage time required to attain a desired level of functionality (i.e., a particular negative score on the 'major storage time-related' component) depends on calcium content, with cheeses having a relatively low content in need of a shorter time and vice versa. Furthermore, Ca-to-protein was found to be the only factor ($P < 0.001$) affecting the variability in the 'minor storage time-related' component (Supplementary Table D).

The effects of the Ca content on the overall functional performance of cheese after different storage times is shown in Figure 6, and demonstrates how the level of Ca content consistently influenced the scorings on the 'major storage time-related' and 'minor storage time-related' components after 2, 8, 16 or 32 d of storage at 4°C, and therefore the functional performance of the cheese. Higher Ca content resulted in firmer cheeses, and lower Ca content in better functional performance of the heated cheese, as demonstrated by higher negative score on the 'major storage time-related'

490 component. Hence, the Ca content is a critical factor modulating functionality and should be tightly
491 controlled to produce cheese with consistent functional performance. The importance of Ca on the
492 functionality of LMPS Mozzarella confirms the findings of previous studies (Guinee et al., 2002;
493 Joshi et al., 2004; Banville et al., 2013).

CONCLUSIONS

LMPS Mozzarella cheese was produced on industrial-scale following a standardized procedure. A total of 7 cheese vats and 75 blocks of cheese were sampled over a 1.5 year period, and characterized for their physicochemical and functional properties after 0, 1, 2, 4, 8, 16 or 32 d of storage at 4°C. The functional performance of the heated cheese developed markedly during the first 16 d, concomitant with a substantial increase in protein hydration, as measured by ^{1}H-NMR relaxometry, and a gradual increase in primary proteolysis (pH4.6SN). Prolonging the storage time to 32 d, resulted in a less significant change in cheese functionality. The level of serum-soluble Ca, expressed as a percentage of total calcium content, mainly affected the firmness of the unheated cheese.

Despite the use of a defined manufacturing procedure, a significant variation in the functional performance of the heated cheese obtained at different production dates was observed.

Principal component analysis (PCA) identified two components, which we denoted as the ‘major storage time-related’ and ‘minor storage time-related’ components. These components represent linear combinations of correlated physiochemical and functional variables, corresponding in the former component to pH4.6SN, cohesiveness, springiness, EW_0, LT_{max}, COT and flow, and in the latter component to pH, soluble Ca, firmness and chewiness. The components, which were a measure for the overall functional performance of the cheese, separated the cheeses on the basis of storage time and production date, and provided insights in the source and degree of the variation between cheeses from different production dates.

A linear model describing the effects of milk composition (protein and fat), pH during manufacturing, and cheese composition (fat-in-dry matter, moisture-non-fat-substances, salt-in-moisture and calcium) on the ‘major storage time-related’ and ‘minor storage time-related’ components identified the variation in calcium content (27.3 to 32.6 mg.g^{-1} protein) as the main

source of the variability in the functionality of the unheated- and heated cheese after different storage times.

Consequently, we recommend tight control of the calcium content of LMPS Mozzarella as a means of ensuring more consistent functionality. High-calcium cheeses (32.6 $mg.g^{-1}$ protein) were firmer and more resistant against heat-induced flow. The effects of a high calcium content on the functional performance of the cheese could, however, be mitigated by prolonging the storage time at 4°C. This finding is of relevance to manufacturers interested in the development of LMPS Mozzarella variants with points of differentiation (e.g., in shreddability, flow or shelf life) for different markets. The methods applied in this study have been insightful both in characterizing the quality of LMPS Mozzarella, and identifying the sources of variability in quality over the cheesemaking season. The cheeses in this study were produced applying a subset of manufacturing conditions that are found in commercial production of Mozzarella (e.g. relatively high pH at whey drainage, high stretching temperature), and were characterized by a relatively high Ca content and low proteolysis. Hence, their application to LPMS Mozzarella in general has to be considered with due consideration of the particular production conditions and composition of the cheese.

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CONFLICTS OF INTEREST

There are no conflicts of interest.

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666 Sci. 76:3664-3673. https://doi.org/10.3168/jds.S0022-0302(93)77708-5.

667 **Table 1. Sampling pattern of blocks per production date and per storage time at 4°C**

Storage time at 4°C (d)[1]	Sampled number of blocks per production date							Total number of blocks per storage time at 4°C
	5/11/2017	8/01/2018	12/03/2018	19/03/2018	18/06/2018	5/11/2018	20/01/2019	
Serum distribution	Vat A	Vat B	Vat C	Vat D	Vat E	Vat F	Vat G	
0	1	1	-	-	-	-	-	2
1	1	1	-	-	-	-	-	2
2	1	1	-	-	-	-	1	3
4	1	1	-	-	-	-	1	3
8	1	1	-	-	-	-	1	3
16	1	1	-	-	-	-	-	2
32	1	-	-	-	-	-	-	1
Other physicochemical[2] and functional properties[3]	Vat A	Vat B	Vat C	Vat D	Vat E	Vat F	Vat G	
2	3	2	2	2	2	2	-	13
4	3	-	-	-	2	-	2	7
8	3	2	2	2	2	-	-	11
16	3	2	2	2	2	2	2	15
32	3	2	-	2	2	2	2	13

668 [1]The physicochemical and functional properties of industrial low-moisture part-skim Mozzarella cheeses were evaluated during storage at 4°C.
669 [2]Other physicochemical properties: pH, serum-soluble Ca and pH 4.6 soluble N.
670 [3]Functional properties: firmness, springiness, cohesiveness and chewiness of the unheated cheese, and cross-over temperature, maximum value of the loss tangent,
671 extension work at 0 or 5 min after melting and flow of the heated cheese.

672 **Table 2. Details of the cheese-making process and the composition of the resultant cheeses obtained at different production dates[1]**

	Vat A	Vat B	Vat C	Vat D	Vat E	Vat F	Vat G	Variability (%)
Production date	5/11/2017	8/01/2018	12/03/2018	19/03/2018	18/06/2018	5/11/2018	20/01/2019	
Milk composition								
Fat (%, wt/wt)	2.84	2.87	2.86	2.86	2.82	2.77	2.76	3.9
Protein (%, wt/wt)	3.73	3.68	3.66	3.64	3.47	3.75	3.78	8.4
Cheese manufacture[2]								
$pH_{renneting}$	6.55	6.54	6.57	6.54	6.49	6.56	6.55	1.2
$pH_{drainage}$	6.36	6.39	6.41	6.35	6.36	6.38	6.37	0.9
$pH_{milling}$	5.19	5.15	5.15	5.11	5.06	5.16	5.17	2.5
Cheese composition[2]								
Dry matter (%, wt/wt)	51.6 ± 0.5^{c}	52.1 ± 0.4^{bc}	52.0 ± 0.3^{bc}	52.1 ± 0.4^{b}	52.1 ± 0.2^{b}	52.9 ± 0.3^{a}	52.0 ± 0.2^{bc}	2.5
FDM (%)	41.8 ± 0.5^{bc}	42.3 ± 0.5^{ab}	41.8 ± 0.5^{bc}	42.7 ± 0.5^{a}	41.4 ± 0.4^{c}	41.4 ± 0.6^{bc}	41.9 ± 0.4^{bc}	3.1
MNFS (%)	61.7 ± 0.6^{a}	$61 .5 \pm 0.3^{abc}$	61.3 ± 0.5^{ab}	61.6 ± 0.6^{ab}	61.1 ± 0.3^{bc}	60.3 ± 0.5^{c}	61.4 ± 0.4^{ab}	2.3
S/M (%)	2.7 ± 0.1^{a}	2.7 ± 0.1^{a}	2.5 ± 0.1^{b}	2.3 ± 0.1^{b}	2.4 ± 0.0^{b}	2.4 ± 0.1^{ab}	2.0 ± 0.3^{c}	28.8
Ca ($mg.g^{-1}$ protein)	32.6 ± 1.4^{a}	31.1 ± 0.6^{ab}	27.3 ± 0.7^{c}	28.5 ± 0.7^{bc}	28.2 ± 1.0^{bc}	30.9 ± 2.9^{ab}	30.4 ± 1.0^{ab}	17.6

673 [1]Presented data for milk composition or cheese manufacture represent one measured value per vat, whereas data for cheese composition represent mean values with
674 standard deviation measured on at least four different cheeses per vat.
675 [2]Abbreviations: pH at rennet addition, $pH_{renneting}$; pH at vat drainage, $pH_{drainage}$; pH at curd milling, $pH_{milling}$; fat-in-dry matter, FDM; moisture-non-fat-substances,
676 MNFS; salt-in-moisture, S/M.
677 [a,b,c]Values in rows with different superscript letters denote a difference at $P < 0.05$.

678 **Table 3. Relationships between physicochemical and functional characteristics of the unheated or**
679 **the heated cheese[1]**

A_{3ms}	df_{model}	df_{error}	F_{ratio}	*P*
Firmness	1	22	1.61	-
Springiness	1	22	2.95	-
Cohesiveness	1	22	5.72	*
Chewiness	1	22	0.27	-
COT	1	22	47.12	***
LT_{max}	1	22	34.44	***
EW_0	1	22	6.61	**
Flow	1	22	23.89	***

pH	df_{model}	df_{error}	F_{ratio}	*P*
Firmness	1	57	2.03	-
Springiness	1	57	1.49	-
Cohesiveness	1	57	2.54	-
Chewiness	1	57	0.15	-
COT	1	57	10.79	**
LT_{max}	1	57	6.83	*
EW_0	1	57	2.98	-
Flow	1	57	3.60	-

SCa	df_{model}	df_{error}	F_{ratio}	*P*
Firmness	1	57	9.34	**
Springiness	1	57	0.03	-
Cohesiveness	1	57	1.39	-
Chewiness	1	57	7.79	**
COT	1	57	1.19	-
LT_{max}	1	57	3.28	-
EW_0	1	57	1.43	-
Flow	1	57	0.91	-

pH4.6SN	df_{model}	df_{error}	F_{ratio}	*P*
Firmness	1	57	3.48	-
Springiness	1	57	36.90	***
Cohesiveness	1	57	55.62	***
Chewiness	1	57	28.66	***
COT	1	57	184.22	***
LT_{max}	1	57	168.34	***
EW_0	1	57	137.22	***
Flow	1	57	31.97	***

680 [1]Abbreviations: less-mobile-serum, A_{3ms}; serum-soluble calcium content, SCa; primary proteolysis, pH4.6SN; cross-
681 over temperature, COT; maximum value of the loss tangent, LT_{max}; extension work at 0 min after melting, EW_0.
682 Simple regression analysis between physicochemical (A_{3ms}, pH, SCa or pH4.6SN) and functional characteristics of the
683 unheated (firmness, springiness, cohesiveness or chewiness) or the heated cheese (COT, LT_{max}, EW_0 or flow) was
684 performed using data obtained on 75 industrial LMPS Mozzarella cheeses. The cheeses were sampled at 7 different
685 production dates, and measured after 0, 1, 2, 4, 8, 16 or 32 d storage at 4°C. The statistical significance (*P*) is given
686 where $P > 0.05$, $P < 0.05$, $P < 0.01$ and $P < 0.001$ are denoted by -, *, ** and ***, respectively.

LIST OF CAPTIONS

Fig. 1 Overall changes during storage at 4°C in serum distribution (A_{3ms}, A_{10ms}, A_{60ms}), cheese pH, serum-soluble Ca (SCa), primary proteolysis (as measured by the level of pH4.6SN), firmness, cross-over temperature (COT), maximum value of the loss tangent (LT_{max}), extension work at 0 or 5 min after melting (EW_0 or EW_5), and flow of commercial LMPS Mozzarella cheeses. The data presented are mean values of seven different production dates, except for those for serum distribution properties, which were measured for three different production dates only (Table 1). Error bars represent the standard deviation.

Fig. 2 Relationships between physicochemical characteristics (less-mobile-serum, A_{3ms}; pH; serum-soluble calcium content, SCa; primary proteolysis, pH4.6SN) and functional characteristics of the unheated (firmness, springiness, cohesiveness) and heated (cross-over temperature, COT; maximum value of the loss tangent, LT_{max}; extension work at 0 min after melting, EW_0; flow) cheeses. The data were obtained from 75 industrial LMPS Mozzarella cheese blocks sampled from seven different production dates, and measured after 0, 1, 2, 4, 8, 16 or 32 d storage at 4°C. Regression lines with 95% confidence limits are shown for significant relationships ($P < 0.05$).

Fig. 3 Loading (A, C) and scores (B, D) plots for LMPS Mozzarella obtained by principle component analysis. Plot A shows the variance in the physicochemical (pH; serum-soluble Ca; pH4.6SN) and functional variables (firmness; springiness; cohesiveness; chewiness; cross-over temperature, COT; maximum value of the loss tangent, LT_{max}; extension work at 0 min after melting, EW_0; and flow) explained by two principal components (PC1 and PC2). Plot C shows the variance in cheese-making related variables (milk fat, milk protein, pH at rennet addition and pH at curd milling) explained by PC1 and PC2. Score plots B and D illustrate the separation of cheeses sampled at different production dates (A, B, C, D, E, F or G) based on their scorings on PC1 and PC2 during storage at 4°C, such that the plots were divided into four quadrants (I, II, III and IV). In plot B, the numbers (0, 2, 4, 8, 16 or 32) correspond to the storage time at 4°C, and the letters (A, B, C, D, E, F or G) to the production date (Table 1).

713

714 **Fig. 4** Changes during storage at 4°C in the scores on the 'major storage time-related' and 'minor storage
715 time-related' components, which represent linear combinations of the physicochemical and functional
716 variables of industrial low-moisture part-skim Mozzarella, and are a measure for the overall functional
717 performance of the cheese. The main variables included in the major (pH4.6SN, cohesiveness, springiness,
718 EW_0, LT_{max}, COT and flow) and minor (pH, soluble Ca, firmness and chewiness) time-dependent
719 components were derived using principal component analysis (Fig. 3). Presented data are mean values of
720 cheeses produced on 7 different production dates; error bars represent the standard deviation.

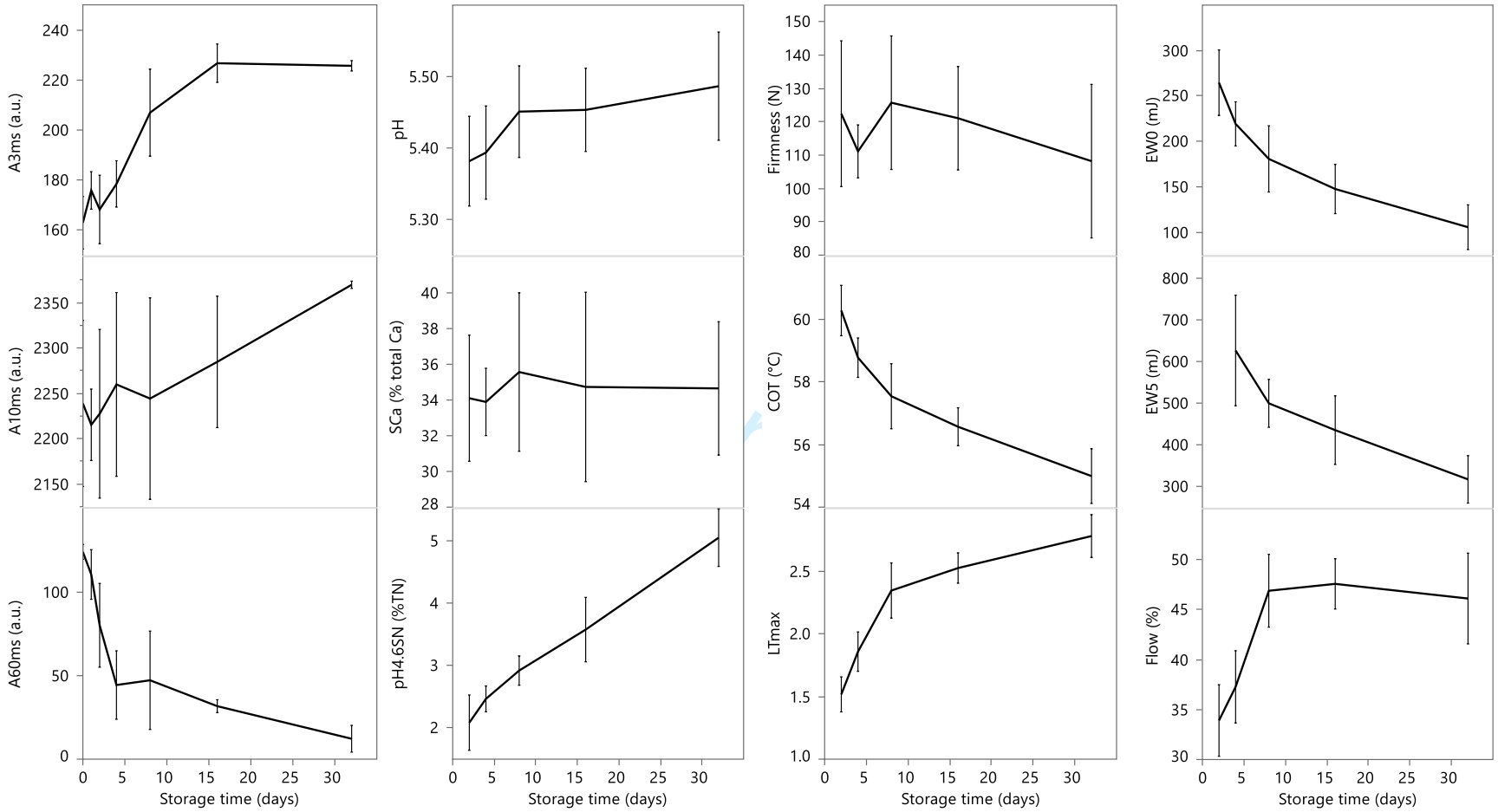
721

722 **Fig. 5** Contour plot for industrial LMPS Mozzarella showing the dependence of storage time at 4°C on
723 calcium content of cheese in attaining a score of -5 to 2 units on the 'major storage time-related' component
724 (linear combination of cheese physicochemical and functional properties: pH4.6SN, cohesiveness,
725 springiness, EW_0, LT_{max}, COT and flow). Each curve or score signifies an overall degree in functional
726 performance of the cheese, as derived from principal component analysis (Fig. 3). Curves with a more
727 negative scoring for the 'major storage time-related component' correspond to cheese with higher values of
728 loss tangent (LT_{max}) and flow of the heated cheese, and lower values for cross-over temperature during
729 heating (COT) and extension work at 0 min after melting (EW_0), and thus are indicative of a more desirable
730 cheese functionality.

731

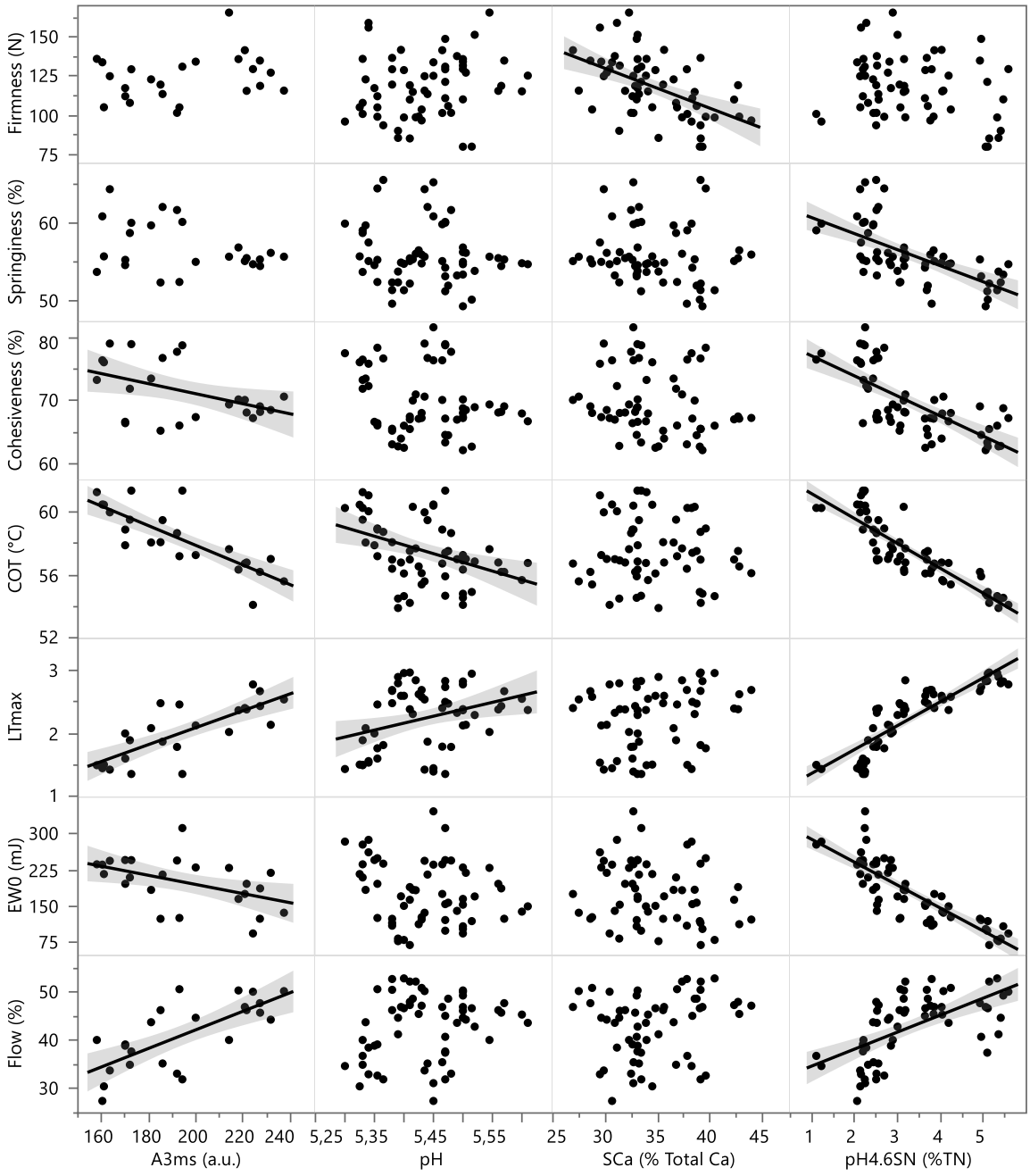
732 **Fig. 6** Scores on the 'major storage time-related' and 'minor storage time-related' components as a function
733 of the variation in calcium content in industrial low-moisture part-skim Mozzarella during 32 days of storage
734 at 4°C. The 'major storage time-related' and 'minor storage time-related' components correspond to linear
735 combinations of functional-performance related variables as derived from principal component analysis,
736 and are a measure for the overall functional performance of industrial low-moisture part-skim Mozzarella
737 cheese. Data illustrated are mean values of cheeses sampled on 7 different production dates. Error bars
738 represent the standard deviation.

To. Fig. 1



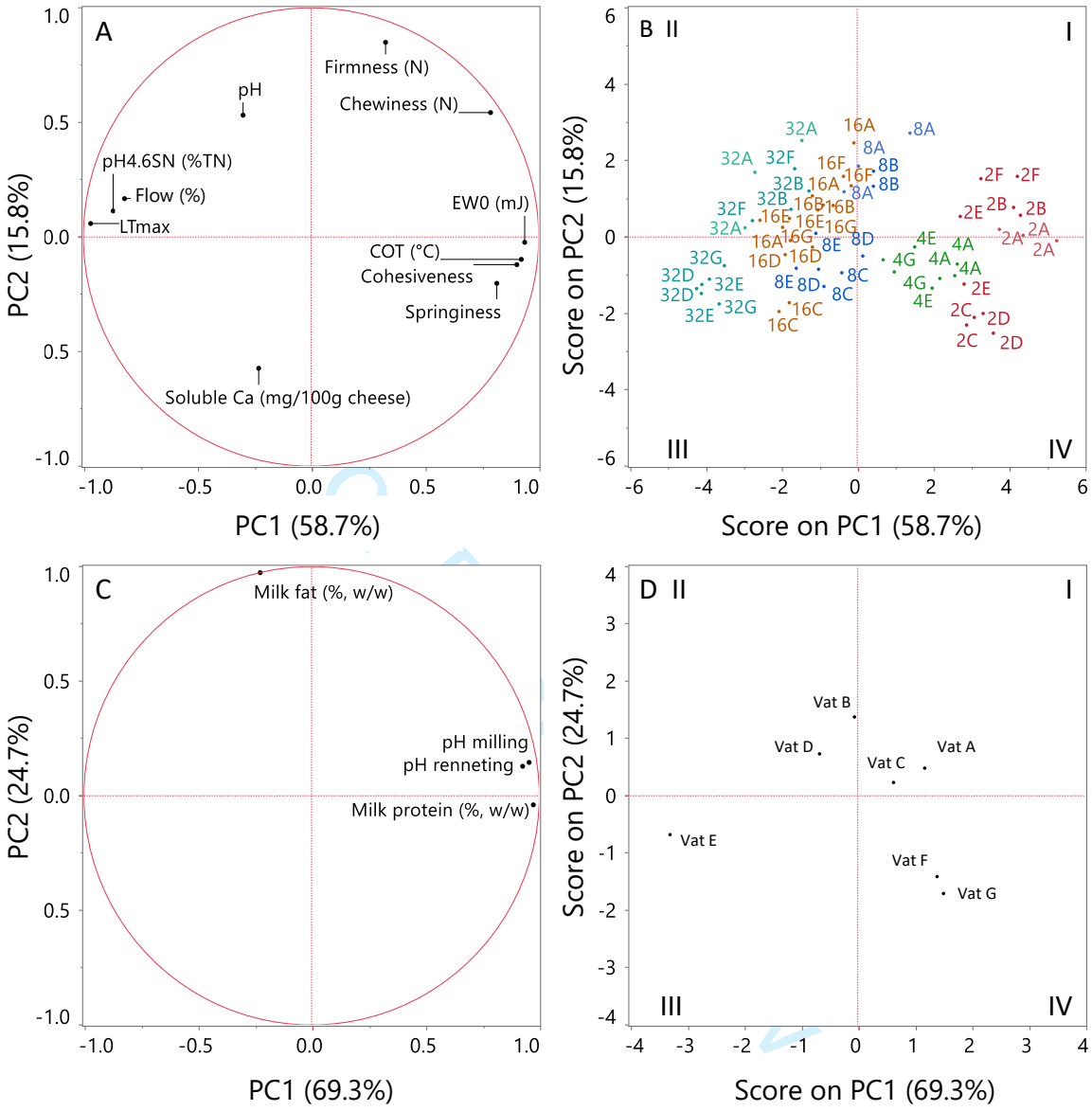
JDS.2020-19047 Fig. 1

To. Fig. 2



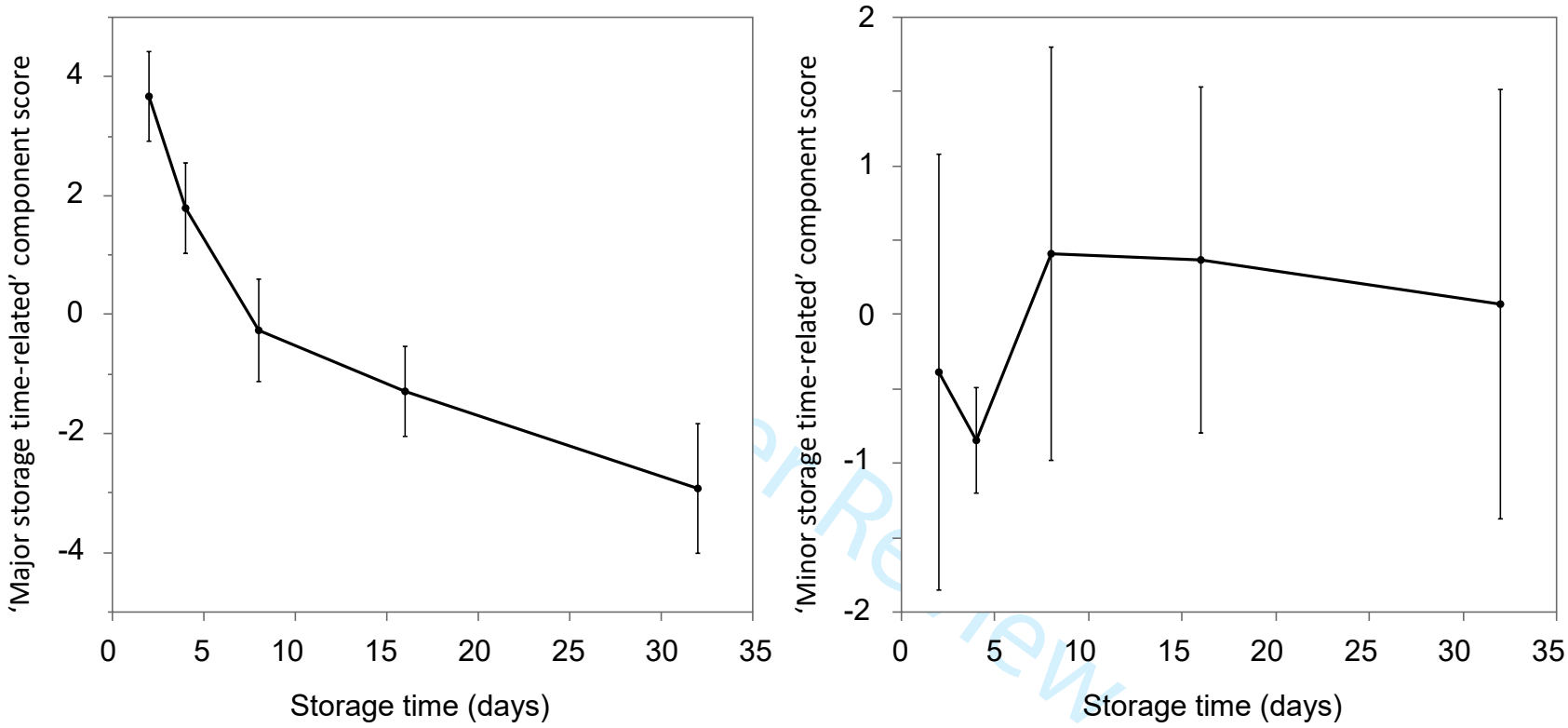
JDS.2020-19047 Fig. 2

To. Fig. 3



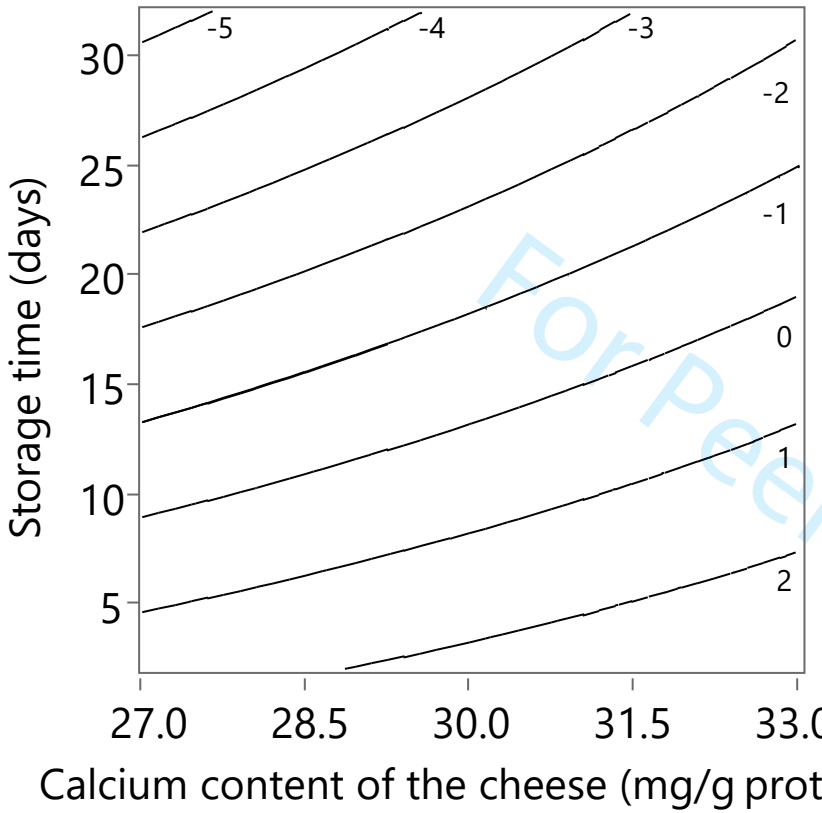
JDS.2020-19047 Fig. 3

To. Fig. 4



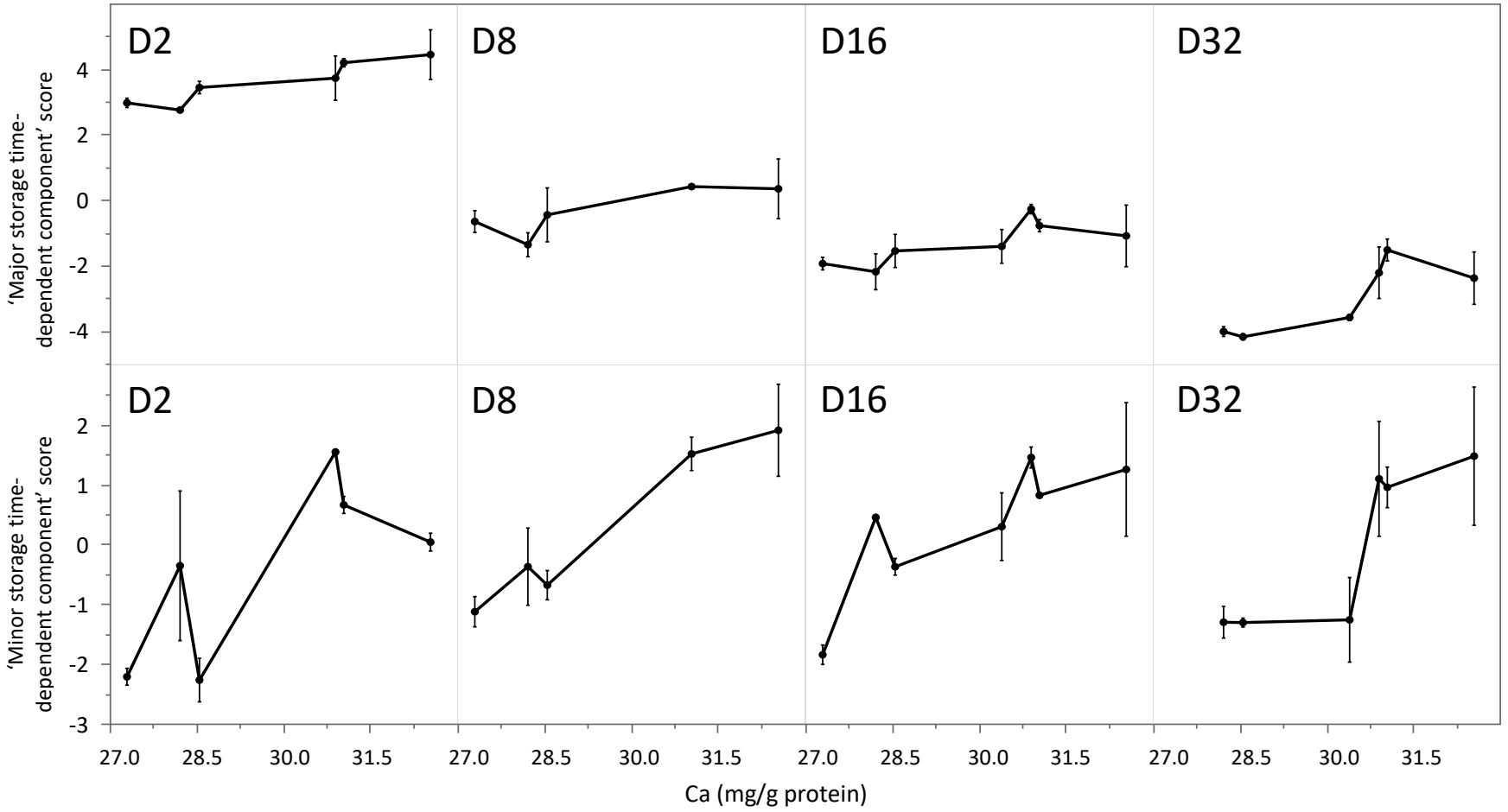
JDS.2020-19047 Fig. 4

To. Fig. 5



JDS.2020-19047 Fig. 5

To. Fig. 6



JDS.2020-19047 Fig. 6

933 # APPENDIX

934 **Supplementary Table A. Effects of storage time at 4°C on the physicochemical and functional properties of industrial low-moisture part-**
935 **skim Mozzarella[1,2]**

		Physicochemical properties						Functional properties						
Vat	Storage time (days)	A_{3ms} (a.u.)	A_{10ms} (a.u.)	A_{60ms} (a.u.)	pH	SCa (%)	pH4.6SN (%TN)	Firmness (N)	Springiness	Cohesiveness	COT (°C)	LT_{max}	EW_0 (mJ)	Flow (%)
A	0	163	2,317	127	-	-	-	-	-	-	-	-	-	-
	1	181	2,241	99	-	-	-	-	-	-	-	-	-	-
	2	160	2,138	49	5.46	33	2.2	129	0.62	0.80	61	1.4	302	34
	4	177	2,175	22	5.46	33	2.5	106	0.61	0.77	59	1.8	233	35
	8	216	2,383	49	5.52	33	3.0	151	0.55	0.70	57	2.2	189	44
	16	229	2,343	32	5.44	28	4.1	120	0.55	0.70	56	2.5	147	49
	32	226	2,370	12	5.52	31	5.3	125	0.55	0.68	55	2.8	109	49
B	0	163	2,161	122	-	-	-	-	-	-	-	-	-	-
	1	171	2,190	122	-	-	-	-	-	-	-	-	-	-
	2	162	2,209	92	5.44	30	2.1	130	0.63	0.78	60	1.4	240	31
	4	170	2,221	61	-	-	-	-	-	-	-	-	-	-
	8	216	2,167	74	5.50	30	2.8	131	0.56	0.68	57	2.1	225	45
	16	224	2,226	31	5.56	32	3.2	117	0.55	0.68	57	2.4	192	46
	32	-	-	-	5.61	34	4.1	120	0.55	0.67	56	2.5	145	45
C	2	-	-	-	5.36	39	2.6	97	0.65	0.78	59	1.8	244	32
	8	-	-	-	5.41	42	2.5	115	0.55	0.67	57	2.4	177	48
	16	-	-	-	5.43	43	3.8	98	0.56	0.67	56	2.7	118	46
D	2	-	-	-	5.32	38	1.2	99	0.59	0.77	60	1.5	281	36
	8	-	-	-	5.42	38	3.2	107	0.56	0.71	59	2.6	184	50
	16	-	-	-	5.47	38	3.7	109	0.53	0.66	57	2.5	156	47
	32	-	-	-	5.51	39	5.1	80	0.50	0.62	55	2.9	111	47
E	2	-	-	-	5.33	34	2.2	121	0.55	0.75	61	1.5	227	35
	4	-	-	-	5.33	37	2.4	116	0.59	0.73	59	2.0	197	39
	10	-	-	-	5.37	36	3.1	112	0.52	0.66	58	2.5	125	48

	16	-	-	-	5.38	38	3.7	133	0.51	0.64	57	2.7	113	52
	32	-	-	-	5.41	40	5.2	92	0.52	0.66	54	3.0	75	53
F	2	-	-	-	5.34	30	2.2	158	0.56	0.74	61	1.5	275	36
	16	-	-	-	5.49	31	2.5	135	0.55	0.68	57	2.4	147	45
	32	-	-	-	5.47	33	5.0	135	0.52	0.64	55	2.8	111	39
G	2	183	2336	100	-	-	-	-	-	-	-	-	-	-
	4	189	2384	50	5.35	33	2.6	115	0.55	0.67	58	1.8	221	39
	8	189	2183	20	-	-	-	-	-	-	-	-	-	-
	16	-	-	-	5.40	35	3.8	111	0.55	0.63	56	2.6	164	47
	32	-	-	-	5.39	33	5.4	88	0.53	0.63	54	2.8	81	43

[1]Abbreviations are: less-mobile-serum fractions, A_{3ms}, A_{10ms}; more-mobile-serum fraction, A_{60ms}; serum-soluble Ca, SCa; cross-over temperature, COT; maximum value of the loss tangent, LT_{max}; extension work at 0 min after melting, EW_0. Data are mean values measured on at least two different cheese blocks per storage time at 4°C except for A_{3ms}, A_{10ms} and A_{60ms}, which were measured on two different locations in one cheese block per storage time.

[2]A cheese vat (coded A, B, C, D, E, F and G) was sampled every two to four months over a period of 16 months (November 2017 - February 2019).

Supplementary Table B. Details of the principal component analyses performed on the cheese-making related variables, and the physicochemical and functional related properties of LMPS Mozzarella[1]

Variables related to	Number of variables	Principal component[2]	Eigenvalue[3]	Cumulative variance (%)	Kaiser-Meyer-Olkin measure of sampling adequacy[4]	Bartlett's Test of sphericity[5]
Physicochemical and functional properties of the cheese	11	1	6.45	58.7	0.644	0.000
		2	1.74	74.5		
		3	0.98	83.3		
Cheese-making	4	1	2.77	69.3	0.659	0.048
		2	0.99	94.0		

[1]Principal component analysis was used to reduce the number of variables by combining them into linear combinations (principal components) on the basis of their correlation, while retaining the highest amount of variance among the studied variables. Principal component analysis was performed when the Kaiser-Meyer-Olkin value was larger than 0.6 and when the Bartlett's test of sphericity returned significant at $P < 0.05$.

[2]The number of principal components was based on Eigenvalues > 1, and the cumulative percentage of the variance explained.

[3]The Eigenvalue is a measure for the amount of variance in the direction of the corresponding principal component.

[4]The Kaiser-Meyer-Olkin test measures the proportion of variance that could be attributed to underlying principle components.

[5]The Bartlett's test of sphericity tests the correlation matrix of the variables of interest against the identity matrix.

Supplementary Table C. Effects of compositional variation, storage time at 4°C and their interactions on the 'major storage time-related' and 'minor storage time-related' components[1]

		'Major storage time-related' component				'Minor storage time-related' component			
Effects of[2]		df	SS	F_{ratio}	*P*	df	SS	F_{ratio}	*P*
'Milk protein-related' component	('MP')	1	277	165	*	1	7	4	*
Storage time	(ST)	1	9.51	5.68	***	1	1.56	0.95	-
Interaction	('MP' x ST)	1	0.36	0.21	-	1	1.41	0.86	-
'Milk fat-related' component	('MF')	1	266	148	-	1	0	0	-
Storage time	(ST)	1	2.81	1.56	***	1	2.19	1.23	-
Interaction	('MF' x ST)	1	0.42	0.23	-	1	1.10	0.62	-
FDM (%)	(FDM)	1	273	148	-	1	2	1	-
Storage time	(ST)	1	0.21	0.12	***	1	2.14	1.21	-
Interaction	(FDM x ST)	1	0.12	0.06	-	1	0.02	0.01	-
MNFS (%)	(MNFS)	1	272	148	-	1	2	1	-
Storage time	(ST)	1	0.11	0.06	***	1	1.89	1.08	-
Interaction	(MNFS x ST)	1	0.35	0.19	-	1	0.87	0.50	-
S/M (%)	(S/M)	1	260	163	**	1	19	14	**
Storage time	(ST)	1	14.37	9.04	***	1	3.41	2.46	-
Interaction	(S/M x ST)	1	0.20	0.13	-	1	1.19	0.86	-
Ca ($mg.g^{-1}$ protein)	(Ca)	1	283	197	***	1	44	44	***
Storage time	(ST)	1	22.43	15.61	***	1	0.66	0.66	-
Interaction	(Ca x ST)	1	2.05	1.43	-	1	2.25	2.26	-

[1]Abbreviations: FDM, fat-in-dry matter; MNFS, moisture-non-fat-substance, S/M, salt-in-moisture.

[2]The factors 'milk protein-related' and 'milk fat-related' components correspond to linear combinations of milk contents of protein and fat, and pH during manufacturing as derived from principal component analysis, whereas the response variables 'major storage time-related' and 'minor storage time-related' components correspond to linear combinations of the physicochemical and functional properties of low-moisture part-skim Mozzarella, and are a measure for the overall functional quality of the cheese. The statistical significance (*P*) for treatment effects on the 'major storage time-related' or 'minor storage time-related' component is given where $P > 0.05$, $P < 0.05$, $P < 0.01$ and $P < 0.001$ are denoted by -, *, ** and ***, respectively.

Supplementary Table D. Linear model describing the effects of calcium-to-protein, salt-in-moisture content, storage time and their interactions on the 'major storage time-related' and 'minor storage time-related' components[1]

'Major storage time-related' component				
Effects test				
Source	df	SS	F_{ratio}	*P*
Storage time (days)	1	269.5	188.1	***
Ca ($mg.g^{-1}$ protein)	1	10.0	7.0	**
Salt-in-moisture (%, wt/wt)	1	2.8	1.9	-
Storage time (days)*Ca ($mg.g^{-1}$ protein)	1	2.5	1.8	-
Storage time (days)*Salt-in-moisture (%, wt/wt)	1	0.7	0.5	-

'Minor storage time-related' component				
Effects test				
Source	df	SS	F_{ratio}	*P*
Storage time (days)	1	1.2	1.3	-
Ca ($mg.g^{-1}$ protein)	1	24.7	25.4	***
Salt-in-moisture (%, wt/wt)	1	1.2	1.2	-
Storage time (days)*Ca ($mg.g^{-1}$ protein)	1	0.3	0.3	-
Storage time (days)*Salt-in-moisture (%, wt/wt)	1	1.5	1.6	-

[1]The response variables 'major storage time-related' and 'minor storage time-related' components correspond to linear combinations of physicochemical and functional properties of low-moisture part-skim Mozzarella as derived from principal component analysis, and are a measure for its overall functional quality. The statistical significance (*P*) for treatment effects on the 'major storage time-related' or 'minor storage time-related' component is given where $P > 0.05$, $P < 0.01$ and $P < 0.001$ are denoted by -, ** and ***, respectively.

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Reporting checklist for JDS for primary research that does not involve animals. Adapted from the REFLECT and STROBE-Vet statements. For intervention trials or observational studies in humans, consider using CONSORT or STROBE.

Indicate where in the paper these items are reported

Paper section and topic	Item	Descriptor of statement item	Reported on Page #
Title & Abstract	1	Describe how experimental units were allocated to treatments (e.g., "random allocation," "randomized," or "randomly assigned"), or whether the study was observational, or an assessment of a method. Clearly state whether the outcome was the result of natural exposure or was the result of a controlled experiment. For observational studies, include a common study design term.	**3**
Introduction Background	2	Provide scientific background and explanation of rationale.	**4-5**
Methods Participants	3	If relevant, describe the eligibility criteria for human participants (e.g., in sensory evaluation or in surveys) and the settings and locations where the data were collected.	**Not relevant**
Interventions	4	Describe precise details of the interventions (treatments) for each group, the level (e.g., farm, animal, batch, or product) at which the intervention was allocated, and how and when interventions were administered.	**6-7; Table 1**
Objectives	5	State specific objectives and hypotheses. Clearly state primary (i.e., the one that determined the sample size) and secondary objectives (if applicable).	**5 lines 91-97**
Outcomes	6	Clearly define primary and secondary outcome measures and the levels at which they were measured, and, when applicable, any methods used to enhance the quality of measurements (e.g., multiple observations, training of assessors).	**Tables 2-3; Figs.**
Sample size	7	Explain how sample size was determined and, when applicable, explain any interim analyses Where relevant, include sample size determinations at each level of the organizational structure and how any non-independence among groups or samples within a group were accounted for.	**6-7; Table 1**
Randomization -- Sequence generation	8	Describe the method used to generate the random allocation scheme, including details of any restrictions (e.g., blocking, stratification)	**Not relevant**
Randomization -- Allocation concealment	9	Describe the method used to implement random allocation, including how treatment assignment was concealed (e.g., treatments may be randomly assigned, but if study units or samples are labelled with letters or colors, differentiation between groups is not concealed).	Not relevant

Reported on page #

Randomization -- Implementation	10	Describe who generated the allocation sequence, who enrolled study units, and who assigned study units to their groups at the relevant level of the organizational structure.	**Not relevant**
Blinding (masking)	11	State whether or not those administering the interventions and those assessing the outcomes were blinded to group assignment; if done, how the success of blinding was evaluated. Provide justification for not using blinding if it was not used.	**Not relevant**
Analytical methods	12	Describe the analytic methods in sufficient detail for replication. Provide data or references to validate the accuracy of the methods under the conditions of this study. Include measures of the precision (repeatability) of assays and the limits of quantification. In sensory evaluations of foods, report the individual traits assessed, not only aggregated scores. For identification, or description of the properties of biological compounds, report the composition of compounds and the techniques by which they were determined. Describe and support how the techniques were validated.	**7-10**
Statistical methods	13	Specify the statistical methods used to compare groups for all outcomes. Clearly state the level of the data on which statistical analysis were performed i.e., show that the correct degrees of freedom were employed for the structure of the data. Clearly state if repeated measures of the outcome were made and how this was accounted for in the statistical analyses. Clearly state all covariates tested.	**10-11**
Results Study flow	14	Account for the flow of study units through each stage of the study and analysis (a diagram is recommended). Specifically, for each group, report the numbers of study units randomly assigned or enrolled, receiving intended treatment, completing the study protocol, and analyzed or excluded. Describe any deviations from the study protocol as planned, and the reasons for these changes.	**7 Lines 134-137;**
Contextual data	15	Where human evaluators (e.g., sensory analysis) or subjects are involved, describe the demographic and other relevant characteristics of participants.	**Not relevant**
Numbers analyzed	16	Specify the number of study units (denominator) in each group included in each analysis and whether the analysis was by "intention-to-treat" or whether units were excluded if they did not comply with the intended treatment. State the results in absolute numbers when feasible (e.g., 10/20, not 50%).	**Table 1**
Outcomes	17	For each primary and secondary outcome, provide a summary of results for each group, accounting where relevant for each relevant level of the organizational structure, and the estimated effect size and its precision (e.g., 95% confidence interval). Where relative measures of effect are reported, also provide absolute values.	**Tables 2-3; Figs.**
Ancillary analyses	18	Address multiplicity by reporting any other analyses performed, including subgroup analyses and adjusted analyses, indicating which were pre-specified and which were exploratory.	**Not relevant**

			Reported on page #
Discussion Interpretation	19	Provide interpretation of the results, taking into account the study hypotheses, and sources of potential bias or imprecision, including explicit discussion of multiplicity of analyses and outcomes. Explicitly discuss the strengths and limitations of the study. Discuss both direction and magnitude of any potential bias. Place the results in the context of relevant literature and state whether or how the findings should change practice.	12-21
Generalizability	20	Discuss generalizability (external validity) of the study findings.	22-23
Transparency	21	List the sources of funding for the work and acknowledge any potential conflicts of interest that the authors have.	23